Bacitracin A. Isolation by Counter Double-Current Distribution and Characterization*

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ABSTRACT: A counter double-current distribution procedure is described which permits the convenient preparation of larger amounts of bacitracin A from commercial bacitracin. Since the procedure requires a significantly smaller number of transfers than regular countercurrent distribution to effect a satisfactory resolution of the bacitracin mixture, an analytical countercurrent distribution carried out on the 2nd International Standard for Bacitracin in 1963 for the World Health Organization is described for comparison. Structure–stability relationships for bacitracin A have been investigated further. The role of ionic zinc in complex formation with bacitracin A was investigated in relation to increased stability of high antibiotic activity. Zinc and bacitracin A combine as a 1:1 complex, whose association constant was determined spectrophotometrically to be 2.5×10^3 at pH 6.34. From data presented, ionic zinc appears to be bound by coordinate bonds to four positions of bacitracin A molecule in the zinc–bacitracin A complex, two on the histidine residue and two on the amino-terminal thiazoline residue. Such binding indicates close spatial proximity of the histidine and terminal thiazoline residue and thus supports the previously postulated conformation of bacitracin A.

Optical rotatory dispersion studies of bacitracin A and its derivatives in the far-ultraviolet region has been further investigated with respect to the conformation of bacitracin A in solution.

Bacitracin is a useful mixture of closely related neutral polypeptide antibiotics from *Bacillus licheniformis.* A commercially available product, bacitracin is used widely as an animal feed supplement in agriculture, but has had a much more limited use in various pharmaceutical preparations. Isolation of individual bacitracins from the commercial bacitracin mixture in more than trace amounts has presented a difficult separation problem which thus far has been solved only by countercurrent distribution separations using a rather large number of transfers (Barry *et al.*, 1948; Craig *et al.*, 1949, 1952, 1958; Newton and Abraham, 1950; Craig and Konigsberg, 1957). The pattern of Figure 1, obtained from an analytical countercurrent distribution of the 2nd International Standard for Bacitracin (zinc bacitracin) carried out in 1963 as a service to the World Health Organization illustrates the detail involved in such a fractionation. The 2nd International Standard for Bacitracin can thus be seen to be a complex mixture of different bacitracin antibiotics, even though it had very high biological activity, 74 μ/mg. Besides its high activity, zinc bacitracin was also chosen for this standard because of its greater stability under less-than-optimum storage conditions.

Bacitracin freshly isolated from the culture broth does not long retain its highest biological activity. The thiazoline ring system of the active bacitracins has barely sufficient stability to permit isolation under the mildest conditions. With ionic zinc the bacitracins form slightly soluble complexes with greater stability than the free bacitracins (Chornock, 1957; Gross, 1954). Although the major features of the structure of bacitracin A (Formula 1) have been established (Craig *et al.*, 1958; Abraham, 1957), much remains to be learned about the subtle transformations such structures are capable of undergoing. Probably no single structural formula can adequately represent the true state of affairs since the known chemistry seems best accounted for by a mixture of tautomeric or resonating forms with intramolecular interactions stabilizing certain forms depending upon pH (Konigsberg *et al.*, 1961) and solvent environment. None of the bacitracin dodecapeptides have been crystallized. Proof that a single individual form has been isolated has depended upon fractionation data obtained by countercurrent distribution and CM-cellulose chromatography and by supporting amino acid analyses and transformation experiments (Craig *et al.*, 1958).

The bacitracin structures are particularly interesting from the standpoint of protein chemistry and the interactions which give individuality to each protein. In contrast to proteins, the bacitracins are relatively small molecules; however, several of their amino acids are those which are of the greatest interest in active site studies in enzymes. They, therefore, appear to be especially desirable models for studying specific interactions of the noncovalent type, provided these interactions can be more precisely documented than has heretofore been possible.

For these reasons and for further biological testing of the individual bacitracins, it appeared desirable to develop a way of separating larger amounts of the individual peptides than the procedure of Figure 1 would allow. Counter double-current distribution (Post and Craig, 1963), a variation of countercurrent distribution, seemed ideal for this purpose. Since commercial pharmaceutical grade bacitracin is relatively inexpensive, percentage yield of a pure product was of secondary importance to isolation of a large quantity of very high purity bacitracin A.

Experimental Section

Countercurrent distribution runs were made in an automatic distribution train containing 1020 tubes (Craig and

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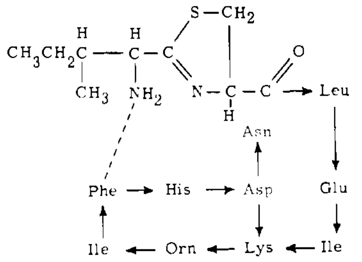

FORMULA 1: Partial structural formula of bacitracin A showing the postulated interaction of the amino-terminal end of the peptide chain with the phenylalanine residue.

King, 1958) of $^5/_3$-ml capacity. Temperature was held constant at 25°. The equilibrated system was made from equal volumes of 1-butanol and 0.5 M sodium phosphate buffer (pH 5.4). A 5-g sample of the 2nd International Standard for Bacitracin was placed in a volume (150 ml of each phase) sufficient to fill the first 50 tubes of the train. A small precipitate at the interface, probably zinc phosphate, was discarded. The settling time for each transfer approximated 3 min. After 1029 transfers, the train was analyzed by absorbance at 252 mμ; the pattern of Figure 1 was obtained.

A large sample from one lot of pharmaceutical grade bacitracin (Commercial Solvents Corp., lot no. 80163-6, 57 μ/mg) served as raw material for the counter double-current distribution experiments. Although this material was not analyzed by countercurrent distribution at the time the counter double-current distribution experiments were carried out, the ultraviolet spectrum was normal for commercial bacitracin preparations showing an absorption maximum at 252 mμ and a weak inflection at 290 mμ. From the absorptivity at 290 mμ, the bacitracin F content was calculated (Codington, 1955) to be 10.1%. From absorptivity at 252 mμ corrected for absorptivity due to bacitracin F content, the combined content of bacitracins A and B was calculated to be 74.4%. Of the remaining 15.5% of the raw material's composition, 6.7% was accounted for as moisture and 8.8% was inert solids which did not absorb ultraviolet energy at these wavelengths. A countercurrent distribution run, however, was made 3 years later on a freshly prepared commercial sample (lot no. 80066-1). Although this material was labeled 65 μ/mg, it assayed 60 μ/mg at about the time the run was made. The pattern for this distribution is shown in Figure 2. The solvent system used in this run was not the same as that of Figure 1, but instead was identical with that used in the counter double-current distribution experiments, 1-butanol–ethyl acetate–0.5 M phosphate buffer (pH 5.43; 70:30:100, v/v). A system with K near 1 for the desired component is best for counter double-current distribution where the desired component is to be retained in the machine. Addition of ethyl acetate to the first system lowered the K to 0.76 for bacitracin A. In this system, however, bacitracin is less soluble than in the system of Figure 1. It was therefore necessary to distribute a 10-g sample equally among the first 100 tubes of the train. The settling time was 4 min.

An automatic counter double-current distribution train with 58 tubes of 50–25-ml capacity was available for use in counter double-current distribution studies with bacitracin. The batchwise method of loading (Post and Craig, 1963) in multiple tubes was used. A 20-g sample of crude bacitracin was added to 960 ml of the upper phase and 750 ml of the lower phase. After the mixture was shaken and allowed to settle several times, a small volume of a third phase separated at the interface. This was discarded. The clear phases were then inserted into the central 30 tubes of the counter double-current distribution train. Those on either side were filled with proper volumes of the appropriate phases of the solvent system.

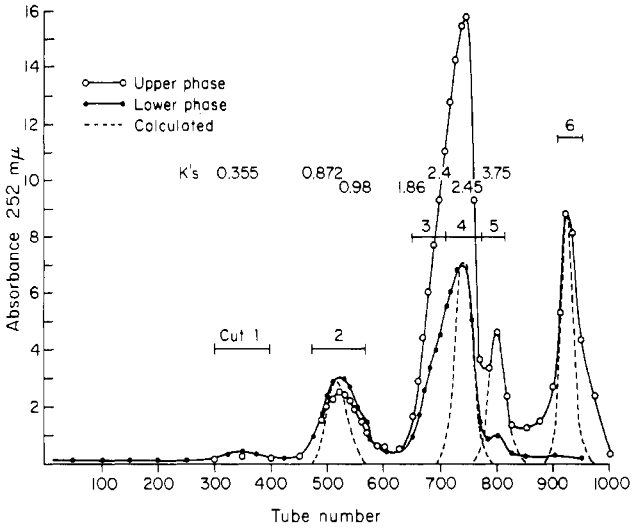

FIGURE 1: Countercurrent distribution pattern at 1029 transfers of the International Bacitracin Standard of 1964. System = 1-butanol–0.5 M phosphate at pH 5.4. Phase volumes = 3:3.

In later runs it was found entirely feasible to start the run with 30 g of crude material and load into 50 tubes of the train.

The 4-min settling time allowed at first could soon be reduced to 2.5 min. From time to time partition ratio, K, determinations were made on the contents of tubes +27 and −27 and tubes +22 and −22 to determine if the major band was moving too rapidly in one or the other direction. The volume of the upper phase introduced on each transfer could be changed to reverse any undesired shifting of the bacitracin A

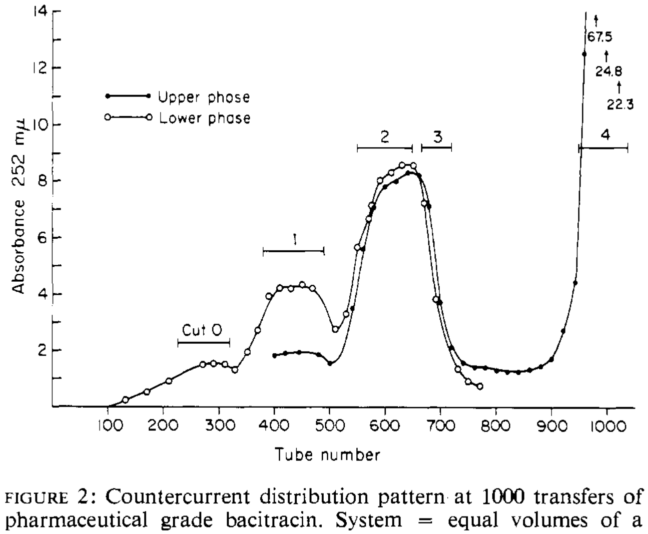

FIGURE 2: Countercurrent distribution pattern at 1000 transfers of pharmaceutical grade bacitracin. System = equal volumes of a mixture of 30% ethyl acetate–70% 1-butanol equilibrated with 0.5 M phosphate buffer at pH 5.43. Phase volumes = 3:3. Cut 1 = B group; cuts 2 + 3 = A group; cut 4 = F group.

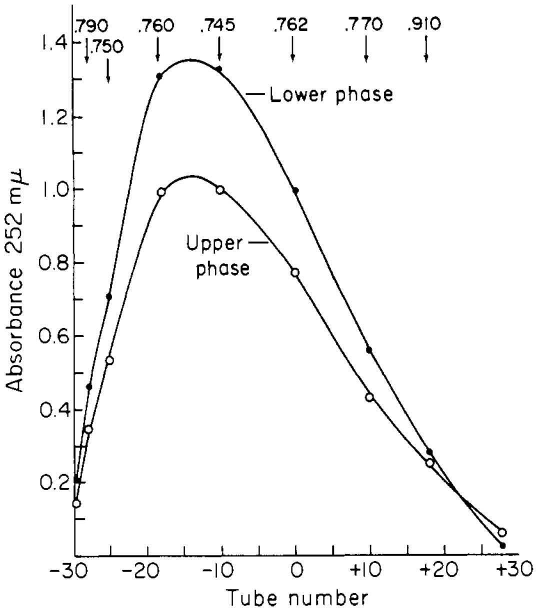

FIGURE 3: Counter double-current distribution patterns of pharmaceutical grade bacitracin. Phase volumes = 32:25. The system is the same as in Figure 2.

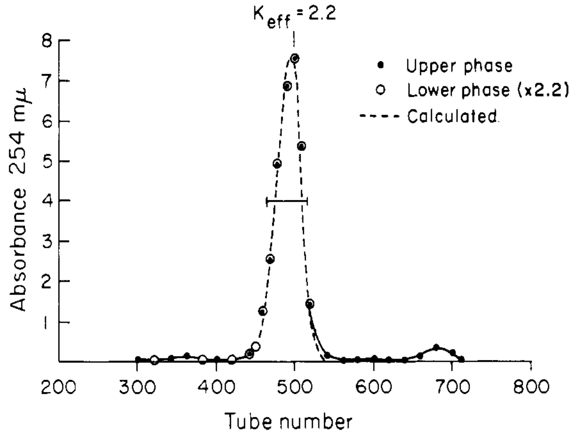

FIGURE 4: Countercurrent distribution pattern at 720 transfers of the recovered bacitracin A from the run of Figure 3. The system is the same as in Figure 1.

band. The object of the control was to keep as much as possible of the band of interest in the train as long as possible.

In a 20-g run after 270 transfers the pattern shown in Figure 3 was obtained. For isolation of bacitracin A only those tubes in which the *K* was constant were combined. *K* determinations were made spectrophotometrically by dilution of a small sample of each phase with 50% methanol until an absorbance in the desired range was obtained on a Beckman Model DU spectrophotometer. From run to run the spread of the cut taken varied somewhat depending upon the exact adjustment of the volume of upper phase and the size of the beginning load.

Bacitracin A was isolated from the combined fractions after counter double-current distribution in the following manner. The upper phase of the cut was separated and set aside. Then to each liter of lower phase 44 g of NaH$_2$PO$_4$ and 5.1 g of Na$_2$HPO$_4$ were added. After the salts had dissolved, the solution was extracted twice with an equal volume of 1-butanol. The butanol extracts were combined with the upper phase above and the butanol was removed on a rotatory evaporator (3-l. flask) at 40° (12 mm) with the addition of water from time to time to prevent precipitation of the bacitracin and to maintain azeotropic conditions for most rapid removal of butanol. The evaporator was fitted with a Kel-F tube for continuous insertion of sample. The solution was never taken to dryness. Evaporation was terminated when the butanol had been entirely removed leaving approximately 200 ml of aqueous solution. The aqueous concentrate was lyophilized immediately. The residue dissolved in water to give a clear colorless solution, pH 5.8. The preparation contained approximately 18% phosphate and lost 6.25% of its weight on drying at 100° *in vacuo*. The preparation assayed 62 μ/mg (82 μ/mg, calculated to a salt free, dry weight basis).

The phosphate content could be considerably reduced by the following procedure. A 1-g sample was dissolved in 10 ml of water and extracted with 20 ml of 1-butanol. The butanol was washed with 10 ml of water and set aside. The first aqueous layer, the volume of which had been reduced to 5 ml, was diluted to 10 ml and reextracted with 20 ml of 1-butanol. The butanol layer was combined with the water wash from the first butanol extract, shaken, and separated. It was necessary to centrifuge the sample at this point to break an emulsion which formed. The butanol layer was combined with the first butanol extract; the butanol was removed by azeotropic distillation with added water in a rotatory evaporator as described above. The final aqueous solution was lyophilized to yield bacitracin A as a white powder which contained 0.46% phosphorus, equivalent to 1.8% NaH$_2$PO$_4$. Bacitracin recovery was of the order of 75–80%, but the aqueous phases could be extracted further to obtain a higher yield if desired.

Seven counter double-current distribution runs carried out as above gave bacitracin A preparations containing approximately 2% NaH$_2$PO$_4$ which consistently assayed near 70 μ/mg. A sample (1 g) of this material was subjected to a 720-transfer analytical countercurrent distribution in the 1-butanol–0.5 M phosphate (pH 5.4) solvent system. The result is shown in Figure 4.

For analytical CM-cellulose chromatography of the bacitracins, a slightly modified variation of a previously described procedure (Konigsberg and Craig, 1959) was used. A borosilicate glass chromatography tube (0.9 × 34 cm) was packed in the conventional manner with preswollen CM-cellulose (Whatman CM 52). The bacitracin A sample (9.0 mg), dissolved in 1.0 ml of 0.05 M sodium acetate buffer (pH 4.52), was introduced at the top of the column. The column was developed at a flow rate of 12 ml/hr under a linear gradient of sodium acetate formed by a two-compartment head of equal volumes. The first compartment contained 150 ml of 0.05 M sodium acetate, and the second contained 150 ml of 1.0 M sodium acetate. The column effluent was monitored at 254 mμ by an ISCO ultraviolet analyzer. Figure 5 presents results of a run with material isolated from cut 2 of Figure 2. Absorptivity of the starting material at 252 mμ indicated it to be about 76% bacitracin, the remainder being inorganic phosphate. The material assayed 50 μ/mg (70 μ/mg when calculated to ash-free basis). The curve (Figure 5) contains two principal peaks and a very small one. The strongest gave the typical ultraviolet spectrum of bacitracin A with maximum at 252 mμ. The ultraviolet spectrum of the smaller peak in the middle at tube 58 was identical with that of bacitracin F; absorptivity at 290-mμ maximum indicated the sample contained 6% bacitracin F. A third smaller peak indicated the presence of a transformation product of bacitracin A; from relative areas under the peaks this component accounts for not more than a few per cent of the sample.

For certain experiments connected with the binding of zinc it was important to have the bacitracin A completely free of phosphate. This was achieved by a short countercurrent distribution run in a five-tube train with the system 1-butanol–water and 5:3 phase volumes. The system was kept below pH 6 by bubbling in CO_2. The sample (500 mg) was loaded in the first tube (0); 14 transfers were applied by the method of "single withdrawal." The nine effluent tubes were combined. After adding 25 ml of water the butanol was extracted with peroxide-free ethyl ether, leaving an aqueous layer, which was then lyophilized. The lyophilized powder which contained about 60–70% of the starting sample was free of phosphate. While a higher yield could be obtained by carrying the distribution further, emulsification slowed the operation too much. The bacitracin A preparation dissolved in water to form a crystal clear, colorless solution, pH 6.8 (the isoelectric pH of bacitracin A).

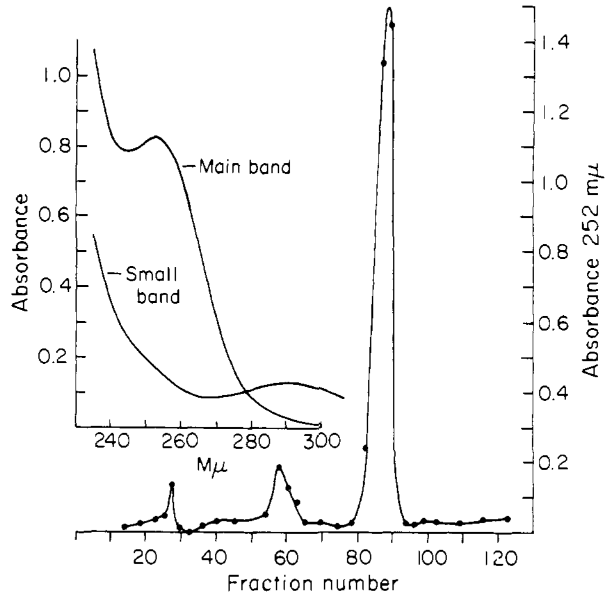

FIGURE 5: Effluent pattern from a CM-cellulose column of purified bacitracin A and absorption spectra of the bands.

Discussion

Bacitracin must be treated very carefully in order to retain maximum antibiotic activity. Commercial bacitracin products are complex mixtures, as demonstrated by the complex countercurrent distribution patterns in Figures 1 and 2. Although the various forms of chromatography have been extensively investigated for the separation of this mixture a measure of success has been achieved only in the case of CM-cellulose chromatography (Konigsberg and Craig, 1959) and this is limited to the purification of only milligram amounts of the individual bacitracins. CM-cellulose chromatography is, however, the best method for analytical separation of the bacitracins since more complete resolution is obtained. Countercurrent distribution is a much better approach for the preparative separation of these particular polypeptides but even a 1000-transfer distribution does not resolve the components completely. However, countercurrent distribution does provide clear-cut group separation of the A, B, C, and F groups, and relatively pure individual preparations can be obtained by selecting appropriate cuts from the overlapping bands. Subsequent distribution can then provide material of the desired purity.

One advantage of liquid–liquid extraction is the ease with which it can be scaled up to larger quantities; scale-up is much easier than with chromatography in spite of statements to the contrary (Novelli, 1968). The counter double-current distribution (Post and Craig, 1963) extension of countercurrent distribution was designed to fractionate large quantities by solvent extraction. A counter double-current distribution train can be used in a variety of ways: in steady-state extraction, in a batch process, or in combinations as desired. To obtain a relatively pure sample of bacitracin A in an amount approximating 20–30 g the batchwise approach appeared to have advantages over the steady state. The latter has been used advantageously to isolate pure fatty acids (Butterfield *et al.*, 1966). In the batch procedure (Post and Craig, 1963) the material of interest could be kept in the train until *K* determinations across the band indicated that a satisfactory purity had been reached for a significant cut.

A number of solvent systems have been used in the countercurrent distribution separation of the bacitracins (Newton and Abraham, 1950; Craig and Konigsberg, 1957). To retain maximum antibiotic activity, the system must be buffered in the range pH 5–6 (Konigsberg and Craig, 1959). Pyridine acetate is the most convenient buffer to use since all components of the system are then volatile and recovery of the material is made much easier. However, after lyophilization from this solvent system bacitracin contains pyridine acetate which, while it appears to have a desirable stabilizing influence, complicates spectroscopic investigations. While phosphate buffers have been found to be satisfactory, removal of phosphate complicates the isolation. The best method for phosphate removal has been found to be a short countercurrent distribution run. In a butanol–water system phosphate strongly favors the aqueous phase and only a few transfers are required to separate it from bacitracin when the pH is near the isoelectric point of the peptide. However, when the buffer is completely removed from the antibiotic, bacitracin A appears to undergo an undesired transformation to F (Craig *et al.*, 1958) at a significantly faster rate and perhaps also an aspartylimidazole transamidation. The two-stage countercurrent distribution described in the Experimental Section left approximately 2% residual phosphate in the bacitracin A. Bacitracin A containing the residual phosphate appeared to be more stable than bacitracin A from which all phosphate had been removed.

In any case bacitracin A prepared in this way from Commercial Solvents bacitracin (lot no. 80163-6) assayed 70 μ/mg without correcting for phosphate or solvent. Upon analytical countercurrent distribution in a 1-butanol–0.5 M sodium phosphate (pH 5.4) system to 720 transfers, bacitracin A prepared as described above had the pattern shown in Figure 4. The weak band at tube 680 due to bacitracin F amounts to less than 2% of the preparation. The bacitracin F must have arisen by transformation of bacitracin A during recovery or storage

TABLE I: Isolation of Bacitracin A by Counter Double-Current Distribution of Commercial Pharmaceutical Grade Bacitracin in a 58-Cell Automatic Machine Using as Solvent System: 1-Butanol–Ethyl Acetate–0.5 M Sodium Phosphate (pH 5.43, 70:30:100, v/v).

					Lyophilized Bacitracin A Isolated				
							After Phosphate Removal		
Counter Double-Current Distribution Run No.	Bacitracin[a] Loaded into Machine (g)	Transfers Applied	Bacitracin A Containing Cuts Recovered		Initial				% Wt Loss on Drying 100° *in vacuo*
			Tubes No.	K_{252} (mμ)	Wt (g)	Assay (μ/mg)	Wt (g)	Assay (μ/mg)	
1	19.9	270	−10 to +10	0.76	4.0[b]	61.5		70[c]	6.4
2	20.0	330	−29 to +29	0.79	5.6	*d*	4.5	70	7.0
3	30.0	318	−25 to +25	0.77	8.0	*d*	7.0	61	
4	26.1	313	−25 to +25	0.79	8.0	*d*	6.1	68	9.9
5	30.0	322	−25 to +25	0.76	7.3	7.3	6.3	69	1.9
6	30.0	334	−25 to +25	*d*	7.5	7.5	6.6	70	9.1
7	30.0	333	−25 to +25	*d*	*e*		7.1	69	10.6

[a] Commercial Solvents Corp., lot 80163-6, 57 IU/mg, 6.7% loss in weight upon drying *in vacuo* at 100°. [b] 18% sodium phosphate; 6.3% loss in weight upon drying *in vacuo* at 100°. [c] Aliquot treated by phosphate removal procedure. [d] Not determined. [e] Phosphate removal procedure applied directly without isolation of crude product.

An even more important conclusion to be drawn from Figure 4 is the fact that *allo*-bacitracin A which broadens the A bands in Figure 1 and Figure 2 has been eliminated by the counter double-current distribution procedure. The optical rotatory dispersion of bacitracin A from counter double-current distribution was practically identical with that reported for pure bacitracin A from countercurrent distribution (Konigsberg and Craig, 1959) thus indicating that no *allo* form was present and no tautomerization had occurred.

Seven batches of bacitracin were processed by counter double-current distribution to bacitracin A as described above (Table I). The final product in all runs except the first were reduced to the 2% phosphate level. While an aliquot of the first run was processed to reduce phosphate concentration to the 2% level, the bulk of the material was left with 18% sodium phosphate present. This latter material assayed 62 μ/mg (equivalent to 81 μ/mg after correction for 18% sodium phosphate and 6.3% moisture). Absorptivity of this material at 252 mμ indicated the presence of 73% bacitracin A (equivalent to 96% on phosphate-and-moisture-free basis). Absorptivity at 294 mμ was a very weak shoulder and indicated the presence of no more than 0.8% bacitracin F. After application of the phosphate removal procedure to an aliquot of the material the resulting product contained 1.9% NaH_2PO_4 and lost 6.5% of its weight on drying *in vacuo* at 100°; assay, 70 μ/mg (equivalent to 77 μ/mg on salt-free, moisture-free basis). Analytical counter double-current distribution of this low-phosphate bacitracin A preparation (Figure 3) showed 2% bacitracin F. CM-cellulose chromatography (Figure 5) indicated presence of the 6% F and not more than a few per cent of another transformation product of bacitracin A. Thus, appreciable amounts of bacitracin A transformation products appear to be formed either upon storage or upon reprocessing. (If the preparation calculates to 77 μ/mg after correction of assay for salt and moisture content, additional correction for the transformation products would make the calculated potency of bacitracin A approximately 83 μ/mg.) The assays of the bacitracin A preparations in Table I after correction for 2% sodium phosphate and moisture content was about 77–79 μ/mg, except for the material from run 3 which calculated to only 66 μ/mg. The cause of the lower activity of bacitracin A from run 3 is not known; however, it is possible that conditions for azeotropic evaporation of 1-butanol were not adhered to as closely as in other runs, especially since the preparation from run 3 was subsequently found to contain about 10% bacitracin F, a transformation product of bacitracin A.

The bacitracin A preparations from counter double-current distribution runs 1, 2, and 4–7 were stored in a deep freeze chest at −20°. The bacitracin A preparation from run 3 was stored at +4° in a refrigerator, and the commercial bacitracin starting material was stored at +10°. The effect of long-term storage on antibiotic activity of these prepartions is shown in Table II. What appears to be a slight trend toward loss of activity may not be statistically significant since the loss is within the limits of accuracy of the analytical method.

CM-cellulose chromatography run under a concentration gradient of sodium acetate (Figure 5) is an excellent way to demonstrate the presence of small percentages of bacitracin F and other transformation products of bacitracin A. The ultraviolet absorption spectrum of peak material from the main band provides further characterization of the bacitracin A. The maximum at 252 mμ in the ultraviolet absorption spectrum of pure bacitracin A from countercurrent distribution peaks has been found to have absorptivity, *A*, 2.52 (for moisture-free product), or *A* 2.00 for the product usually obtained by lyophilization without correction for moisture on solvent, (Konigsberg and Craig, 1959) when measured in water solution. Bacitracin F has *A* 3.59 at 290-mμ maximum; it also ab-

TABLE II: Low-Temperature Stability of Lyophilized Bacitracin A Isolated by Counter Double-Current Distribution and of Spray-Dried Pharmaceutical Bacitracin Starting Material.

		Microbiological Assay (units/mg)[a]			
Bacitracin Preparation	Storage Temp (°C)	0 Months	11 Months	24 Months	41 Months
Commercial, CSC 80163-6	+10	57		57	55
A, counter double-current distribution run 1	−20	62			55[b]
A, counter double-current distribution run 2	−20	70	67	63	68
A, counter double-current distribution run 3	+4	61			62
A, counter double-current distribution run 4	−20	68	62	60	65
A, counter double-current distribution run 5	−20	69	71	67	73
A, counter double-current distribution run 6	−20	70	70	67	68
A, counter double-current distribution run 7	−20	69	68	70	67

[a] Not corrected for moisture or ash. [b] 43 months.

sorbs at 252 mμ (although not a maximum). A_{252} 2.52 (the same as for bacitracin A; Craig *et al.*, 1952). Using absorptivity of bacitracin F and $A_{290\ \mathrm{m}\mu}$ 0.060 from data of Figure 5, the sample content of bacitracin F can be calculated (Codington, 1955) to be 1.6%. Absorptivity at 290 mμ is therefore most useful in revealing the presence of small amounts of bacitracin F in bacitracin A preparations.

The characteristic optical rotatory dispersion of bacitracin A is most interesting in the far-ultraviolet region. More recent results with the Cary spectropolarimeter are shown in Figure 6. Two Cotton effects seem to be present, one in the region of 250 mμ and the other in the region of 220 mμ. It is logical to suspect that the one at 250 mμ results from the thiazoline ring system and, from the behavior of the cyclic antibiotics, gramicidin S-A and tyrocidine B (Ruttenberg *et al.*, 1965; Craig, 1968), that the one in the 220-mμ region results from the larger ring system in bacitracin. It is clearly shown that the former is correct by the type of rotatory dispersion shown by the oxidized bacitracins (Figure 6) in which the thiazoline ring has been eliminated. These derivatives show no absorption band or Cotton effect in the 250-mμ region. Only the negative Cotton effect in the 220-mμ region remains.

On the other hand, bacitracin F retains the second Cotton effect but at a different wavelength than does bacitracin A. The negative specific rotation of the minimum at 230 mμ is reduced by about 50%. In bacitracin F the thiazoline ring has been converted into a thiazole (Konigsberg and Craig, 1962) and the amino group of the N-terminal isoleucine has been converted into a ketone. Thus, the optical activity of two carbon atoms has been eliminated. The Cotton effect at the longer wavelength must be related to the β-carbon atom of the original isoleucine residue which has become α to the keto group.

The rotatory dispersion curve observed for bacitracin A is not greatly altered by a change of solvent, change of pH between 2 and 7, or by 5 M guanidinium chloride. It is, however, shifted by ionic zinc. This is not surprising since bacitracin is known to complex with zinc (Chornock, 1957; Gross, 1954; Garbutt *et al.*, 1961). The effect of the zinc ion on the rotatory dispersion of bacitracin A is shown in Figure 7.

Strong acid has an interesting effect on the rotatory dispersion (Figure 7). In 6 N HCl the positive Cotton effect is shifted to a longer wavelength. This is in accord with the earlier finding (Konigsberg and Craig, 1959) that the 252-mμ absorption band that relates to the thiazoline ring system is shifted to 270 mμ in 6 N HCl. In addition the strength of the minimum in the optical rotatory dispersion is weakened again to about 50% of that at neutral pH.

On the basis of chemical evidence it has been postulated that the terminal isoleucine residue which forms part of the thiazoline ring system is in some way bound to the phenylalanine residue (Formula 1). This interaction would be disturbed by strong HCl and by oxidation of the thiazoline ring to either the sulfonic acid or the thiazole. In these three cases (Figures 6 and 7) the minimum at 228–230 mμ is reduced by about the same amount. On the other hand, complex formation with ionic zinc makes the minimum more strongly negative which may result from a strengthening of the interaction. The thiazoline ring can be shown to be required for complexing with zinc because the optical rotatory dispersion curve

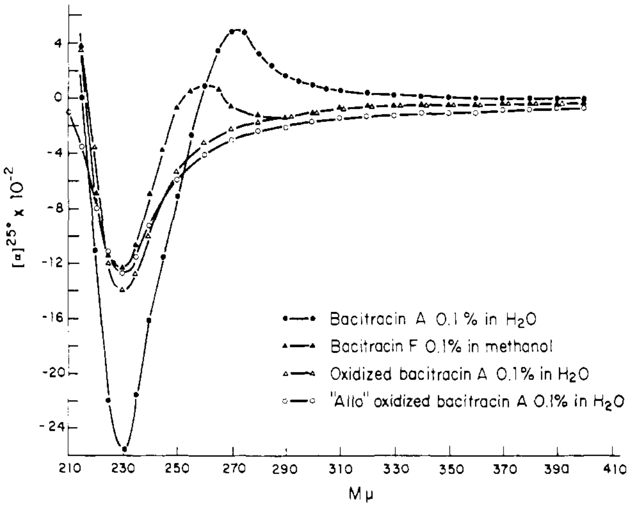

FIGURE 6: Optical rotatory dispersion curves of bacitracin A and derivatives.

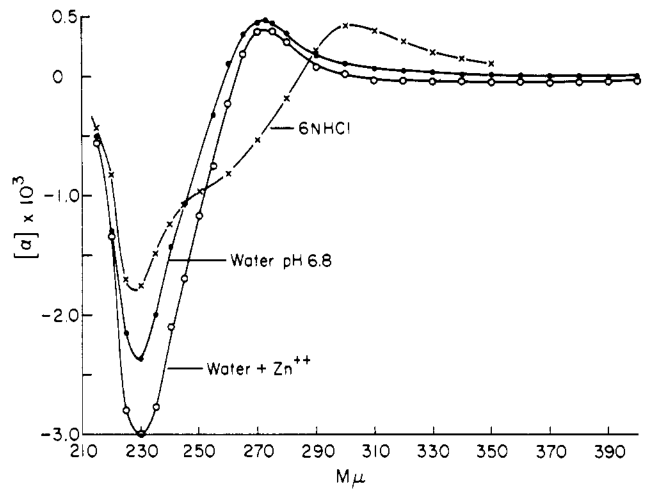

FIGURE 7: Optical rotatory dispersion curves of bacitracin A in different solvents.

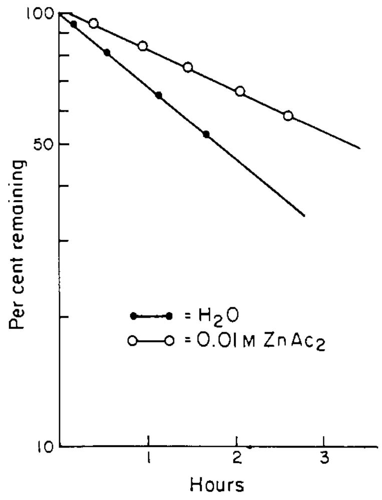

FIGURE 8: Thin-film escape patterns of bacitracin A in water and in zinc acetate solution. Temperature is 25°. Initial concentration of bacitracin is 0.3%.

of oxidized bacitracin is not shifted by zinc. The imidazole of the histidine residue would certainly be involved in the zinc complex (Chornock, 1957; Gross, 1954), a view supported by the fact that below pH 4 the complex does not form and by the known behavior of imidazole (Bradshaw *et al.*, 1968).

It seemed of interest to investigate the binding of the Zn^{2+} ion somewhat further. The thin-film dialysis method (Craig, 1964) should be helpful in this connection. It was found that solid zinc bacitracin A, although soluble in water only to a limited extent, gave an escape rate indistinguishable from that of bacitracin A at the same concentration. However, when the dialysis was carried out against an aqueous solution containing excess zinc acetate (0.01 M), a difference was found as shown in Figure 8. Thus, the zinc complex of bacitracin apparently is largely dissociated in water at the concentration studied. In this study it was also observed that the absorbance of bacitracin A at 252 mμ increased with the addition of zinc acetate to the bacitracin solution. Moreover, the increase of absorbance was a function of the excess amount of zinc acetate added as shown in Figure 9. Obviously the thiazoline ring system is involved in the binding of Zn^{2+}.

Bacitracin A dissolved readily in water to give a clear, colorless solution, pH 6.4. Upon addition of zinc acetate, the pH dropped, indicating liberation of protons. Although the absorptivity of bacitracin A at 252 mμ in water alone is rather insensitive to change in hydrogen ion concentration over the region pH 4–7, the presence of Zn^{2+} changes this behavior. Moreover, a considerably higher absorptivity is observed at pH 6.4 than at pH 4. Thus the binding of Zn^{2+} by bacitracin A is strongly pH dependent.

When, however, $[H^+]$ was held constant at pH 6.35 by addition of NaOH, less Zn^{2+} was required to reach the maximum absorptivity. At 252 mμ a 2.28×10^{-4} M solution of bacitracin A in water, pH 6.35, 25°, had A 0.660. This absorbance increased to 1.110 upon adding 12 molar equiv of zinc acetate and did not change appreciably upon increasing the zinc concentration by 100%. From this, it was assumed that the absorbance of a 2.28×10^{-4} M solution of zinc bacitracin at 252 mμ was 1.110; hence, an absorbance increase of 0.450 occurs at 252 mμ when bacitracin A complexes with Zn^{2+}. This change permits calculation of the association constant, K_a, when the increase in absorbance at 252 mμ is measured against appropriate molar equivalents of Zn^{2+}. These data can be treated best as shown in Figure 10 by the well-known Scatchard plot (Scatchard, 1949). From these data it can be derived that zinc bacitracin A has $K_a = 2.5 \times 10^3$ at pH 6.34.

Since the extrapolated curve of Figure 10 intersects the abscissa near 1, 1 mole of bacitracin A binds 1 mole of zinc; elemental analysis of solid zinc bacitracin (Chornock, 1957; Gross, 1954) supports this conclusion.

Zinc ion is known to have four coordination sites and to complex with four imidazole molecules (Koltun *et al.*, 1959) with association constants ranging from 95 to 420. The pH dependency in the case of bacitracin A–ionic zinc interaction is consistent with the imidazole of the histidine being involved, as was previously concluded from titration studies (Garbutt *et al.*, 1961), and the absorbance increase at 252 mμ indicates the thiazoline group is also involved. Although the thin-film dialysis data at first seemed to indicate a molecular size of the complex larger than uncomplexed bacitracin A, further investigation demonstrated that sodium acetate or ammonium acetate at pH 6.35 produced the same effect on the dialysis rate as did zinc acetate. On the other hand, neither sodium acetate nor ammonium acetate altered the absorbance at 252 mμ. The dialysis result with Zn^{2+} probably reflects a change in the conformation of the bacitracin A molecule caused by a change in the ionic strength of the aqueous solution. Since elemental analysis of solid zinc bacitracin A indicates a zinc to peptide ratio of 1:1, it can be concluded from the thin-film dialysis data of Figure 8 that the zinc–bacitracin A complex is monomeric with a diffusional size not much larger than free bacitracin A. The thiazoline ring system and the histidine residue must therefore be located spatially quite near one another. This supports the previously postulated conformation shown in Formula 1. The data obtained thus far suggest that the single atom of zinc coordinates through four points of attachment to the bacitracin A molecule: the imidazole of the histidine residue, the peptide nitrogen of the histidine residue, the amino group of the N-terminal isoleucine residue, and the nitrogen of the thiazoline ring (or possibly the sulfur). The latter nitrogens are similar to ethylenediamine as regards their distance

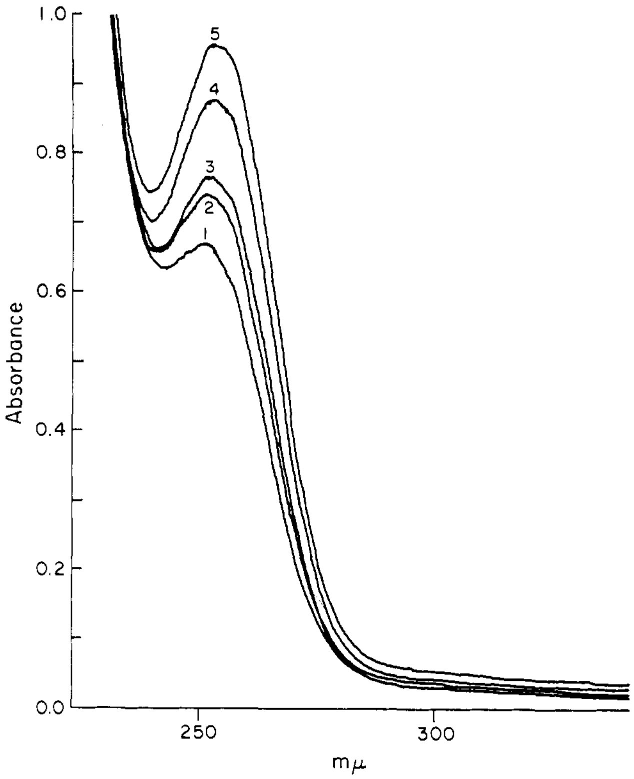

FIGURE 9: Absorption spectra of bacitracin A in different molar equivalents of zinc acetate in water. Curve 1 = no zinc; curve 2 = 1; curve 3 = 2; curve 4 = 6; and curve 5 = 12 equiv.

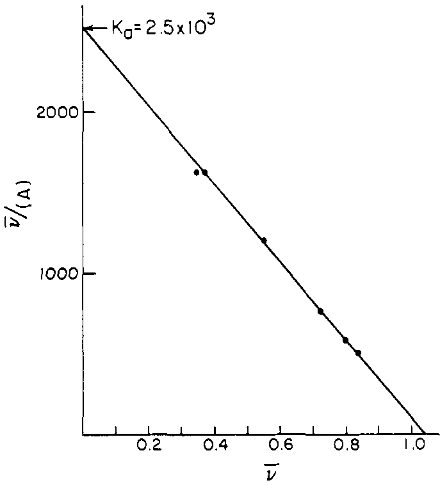

FIGURE 10: Scatchard plot of the binding of zinc ion to bacitracin. ν = ratio of moles of bacitracin bound to moles of bacitracin. (A) = molar concentration of Zn^{2+}.

apart. The possibility that the two free carboxyl groups and the free amino group of the ornithine residue could also react with ionic zinc must be considered; however, the amount of NaOH required to restore the solution to pH 6.34 after the zinc acetate was added to bacitracin A was consistent with the release of approximately four protons.

The above conclusions were confirmed by experiments to be described in a future publication by K. Stewart and L. C. Craig. Excess zinc acetate at pH 6.35 was found to protect the amino-terminal and the imidazole groups from reaction with fluorodinitrobenzene. This contrasted with the previous experience (Craig and Konigsberg, 1957) and with parallel experience with unprotected bacitracin. The zinc did not protect the amino group of the ornithine residue.

The above interpretation is based partly on the experience of Gurd and his collaborators on the binding of copper and zinc by peptides of histidine. Thus with the protected peptide, carbobenzoxy-L-prolyl-L-histidylglycinamide (Koltun *et al.*, 1959), 1 mole of zinc complexes with 4 moles of the peptide. The log K_1 is 2.16. With the peptide Asp-Thr-His-Lys (Bradshaw *et al.*, 1968), Cu^{2+}, which also binds four imidazoles, complexes with only 1 mole of the peptide and in doing so in the region of pH 5–6 releases four protons. It does not complex below pH 4. The points of attachment seem to be the terminal amino group, the amide nitrogen of the threonine residue, the amide nitrogen of the histidine residue, and the N_1 of the histidine imidazole ring. The amino and the carboxyl group of the lysine do not appear to play a role.

Since the instability of bacitracin appears to involve the unexplained transformation of bacitracin A to bacitracin F, it is interesting that the zinc complex of bacitracin is more stable than free bacitracin (Chornock, 1957; Gross, 1954). Since the unstable thiazoline part of the bacitracin A molecule is involved in its transformation to bacitracin F, it is a special point of interest that the data here indicate the thiazoline grouping to be bound to zinc in zinc bacitracin A. Although copper also binds bacitracin (Garbutt *et al.*, 1961), it does not stabilize the antibiotic because it also acts as a catalyst in the oxidation of bacitracin A to bacitracin F by oxygen at higher pH (Sharp *et al.*, 1949). Further investigation of this interesting point is now in progress.

Previous data have indicated that the carboxy-terminal asparagine residue is in some way associated with the thiazoline (Craig *et al.*, 1958). The presence of the second bacitracin A transformation product in preparations isolated in counter double-current distribution suggests that aspartyl → imidazole transamidation (Konigsberg and Craig, 1959) may also be involved in the inactivation of bacitracin A during isolation. Recent data have been reported which indicate that the carboxyl-terminal asparagine may not be dangling, as indicated in Formula 1, but be an integral part of the large polypeptide ring (Ressler and Kashelikar, 1966); however, confirmation of these interesting results have not yet been reported from other laboratories.

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